THE PHYSICOCHEMICAL AND ANTIOXIDANT PROPERTIES OF DOUBLE-BOILED *KELULUT* HONEY

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For my beloved mother, father, family and friends

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ABSTRACT

Kelulut honey is a unique Malaysian food that has beneficial components and high potential in many health aspects. In commercial processing plant, honey is usually subjected to thermal treatment, where its quality is compromised due to unstable and thermolabile components. Therefore, double boiling was chosen as it does not exert significant heat, thus minimizes damage done. The quality was determined after treated by double boiling technique at optimized condition. Three different sources of *Kelulut* honey based on the main types of foods consumed by the bees (Coconut, Multifloral and Wild Flowers) were tested. *Kelulut* honey samples were subjected to an optimized condition at 60 °C for 35 minutes. Thermal treatment at the same condition was conducted for comparison purpose. The results showed that double boiling treatment increased the total phenolic content of *Kelulut-C* honey significantly, from 2430.78 ± 12.24 to 2711.10 ± 23.00 mg GAE/100 g. The treatment also significantly increased the brown pigment for both *Kelulut-C* and *Kelulut-W* honey by 11.11 ± 0.00% and 27.27 ± 0.00%, respectively. Whereas, the pH and moisture content of all samples remain unchanged with no formation of hydroxymethylfurfural (HMF) was observed. A preliminary four-week storage study showed that double boiling treatment improves physicochemical properties of *Kelulut* honey as compared to the untreated samples for all three types of *Kelulut* honey tested. Meanwhile, a room temperature (27 °C) condition leads to an improvement of antioxidant properties as compared to cold temperature (4 °C). In conclusion, double boiling treatment has established itself as a good alternative processing method for *Kelulut* honey, hence increasing the possibility to bring forward *Kelulut* honey as a unique source for the application in food and beverages industries.

ABSTRAK

Madu *Kelulut* merupakan makanan yang unik di Malaysia dengan komponen-komponen berkhasiat dan potensi yang tinggi dalam banyak aspek kesihatan. Di dalam bidang pemprosesan secara komersial, madu selalunya melalui rawatan terma yang menjejaskan kualitinya menerusi komponen yang tidak stabil dan termolabil. Maka, rawatan didihan berganda (*double boiling*) dipilih kerana ia tidak mengeluarkan haba yang signifikan dan mengurangkan kemerosatan kualiti. Kualiti madu akan dikaji dengan menguji kesan rawatan didihan berganda pada kondisi teroptimum ke atas ciri-ciri fizikokimia dan antiokisda untuk tiga sumber (Kelapa, Pelbagai Bunga dan Bunga Liar) madu *Kelulut* yang dinamakan berdasarkan makanan utama lebah tersebut. Sampel madu didedahkan kepada didihan berganda pada kondisi teroptimum (60 °C untuk 35 minit) dengan masa pegangan selama 5 minit (yang diperoleh di awal kajian). Rawatan terma dengan kondisi yang sama turut dijalankan bagi tujuan perbandingan. Hasil menunjukkan bahawa didihan berganda telah meningkatkan kandungan total fenolik madu Kelapa dari 2430.78 ± 12.24 kepada 2711.10 ± 23.00 mg GAE/100 g. Tambahan lagi, rawatan ini juga meningkatkan pigmen perang untuk *Kelulut*-C dan *Kelulut*- W sebanyak 11.11 ± 0.00% dan 27.27 ± 0.00%. Nilai pH dan kandungan kelembapan semua sampel pula berjaya dikekalkan. Tiada penghasilan hidroksimetilfurfural (HMF) ditemui di dalam madu *Kelulut* yang dirawat. Kajian awal penyimpanan selama empat minggu menunjukkan bahawa didihan berganda menambahbaik ciri-ciri fizikokimia ketiga-tiga jenis madu *Kelulut* berbanding sampel yang tidak dirawat. Manakala, suhu bilik (27 °C) memberi kesan peningkatan ciri-ciri antioksida berbanding dengan suhu sejuk (4 °C). Kesimpulannya, didihan berganda mampu menjadi sebuah alternatif kepada rawatan terma konvensional dalam pemprosesan madu *Kelulut*. Didihan berganda boleh diketengahkan untuk penggunaan lanjut madu *Kelulut* sebagai sumber unik dalam industri makanan dan minuman.

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LIST OF SYMBOLS AND ABBREVIATIONS

AA (%)	-	Antioxidant activity in percentage
Abs	-	Absorbance reading
$Abs_{control}$	-	Absorbance reading of the control
Abs_{sample}	-	Absorbance reading of the sample
ANOVA	-	One-way Analysis of Variance
AU	-	Absorbance unit
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
G	-	Gram
HMF	-	Hydroxymethylfurfural
HSD	-	Honestly Significant Difference
L	-	Litre
M	-	Meters
mm	-	Millimeters
Min	-	Minutes
ml	-	Millilitre
nm	-	Nanometer (wavelength)

pH	-	Decadic logarithm of acid dissociation
RSM	-	Response surface methodology
TCD	-	Total color difference
TPC	-	Total phenolic content
TSS	-	Total soluble solids
UV	-	Ultraviolet
UV-Vis	-	Ultraviolet-visible spectroscopy
°C	-	Degree centrigrade
<	-	Less than
>	-	Greater than
±	-	Plus minus
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Kelulut honey is a unique honey from a stingless bee species from Malaysia. The demand on *Kelulut* honey is on the rise for its proclaimed nutritional values, with a distinct taste, flavor and aroma depending on its floral sources (Singh *et al*., 2012). Previous studies have shown that physicochemical properties and quality of honey may vary depending on geographical locations (Gulfraz *et al*., 2011). The relevance of the study has become practical as consumers' interest in quality and benefits of the *Kelulut* honey has increased. Consequently, a more thorough study on the *Kelulut* honey should be conducted regarding all aspect of *Kelulut* honey.

In general, honey is primarily a carbohydrate material with 85% of fructose and glucose and 10% of other complex sugars (Doner, 2000). It is known that these compounds are heat sensitive. The application of conventional thermal treatment in commercial honey processing plants are known to be detrimental to physicochemical and nutritional properties due to the unstable and thermolabile component when heated at more than 60 °C (Akhmazillah, Farid, and Silva, 2013). Previous studies revealed that the qualities of honey such as nutritional value, texture and taste were compromised when it was subjected to thermal treatment (Akhmazillah, Farid, and Silva, 2013; Akhmazillah and Farid, 2015).

For maintaining qualities and properties of honey, there are several methods developed such as ultrasonic (Chong, Chin, and Yusof, 2017), high pressure processing (HPP) (Akhmazilah, Farid, and Silva, 2014) and microwave treatment (Hebbar, Nandini, Lakshmi, and Subramaniam, 2003; Dranca and Oroian, 2013;

Stanisław Kowalski, 2013). These methods aim to inactivate the spoilage microorganisms and undesirable enzyme presence in the products, without destroying or minimal impairing the sensorial attributes, color, flavor, nutritional values and antioxidant activity (Chaikham, Kemsawasd, and Apichartsrangkoon, 2016). Despite the increasing number of studies on quality of honey as affected by thermal and non-thermal process, there is yet a study on the effect of double boiling treatment on the quality of *Kelulut* honey, specifically harvested from different floral sources. Conventional thermal processing in industries can be detrimental to the quality of *Kelulut* honey. Thus, indirect thermal approach, particularly the double boiling treatment can be used as an alternative to maintain the high quality of *Kelulut* honey. Double boiling treatment is extensively practiced by local beekeepers and honey producers in Malaysia.

Therefore, this work aims to investigate the effectiveness of double boiling in maintaining and/or improving the physicochemical and antioxidant properties of *Kelulut* honey tested. The findings obtained from this work may contribute not only for further studies and investigation on *Kelulut* honey, but also provide a scientific information or proven for *Kelulut* honey producers as well as consumers, which then give an impact on extensive use of *Kelulut* honey in food and beverage industry.

1.2 Problem Statement

Information is easily obtained in this millennial era. This includes the information on foods that are being consumed. Hence, consumers are now more alert of what they eat and put on or in their body more than it was before.

This also has affected honey industry includes *Kelulut* honey. Consumers nowadays prefer a high quality and non-modified products, whereas beekeepers prefer a simple and cheaper processing techniques to maintain the quality of *Kelulut* honey. This scenario hence increased the interest on double boiling treatment in honey processing. Conventionally, thermal treatment is the most used method used in industry. However, its application may result in deterioration of thermolabile components (Akhmazillah, Farid, and Silva, 2013). Meanwhile, other processing techniques such as high-pressure, microwave and ultrasonic processing are more expensive and the setups are rather difficult.

One of the pioneers in *Kelulut* beekeeping, Mr. Shamsul from Kampung Bintang, Johor has stated that; most of local beekeepers are using double boiling treatment as conventional practice in *Kelulut* processing. This method has been chosen because it is cheaper, easier to conduct and able to slow down the fermentation reaction. Generally, double boiling technique is known to be an alternative processing method for *Kelulut* honey due to its indirect heat application as compared to conventional thermal treatment. However, the effectiveness of this method was not yet proven scientifically.

Besides the processing method, different floral sources fed by the stingless bees may contribute to different chemical contents. By studying the effect of double boiling treatment on the properties (physicochemical and antioxidant) of different floral sources of *Kelulut* honey, it will aid the *Kelulut* honey as a unique source and its diversification in the food and beverages industry, besides gives clear understanding and provides scientific information not only to scientists but also to the beekeepers and consumers.

1.3 Research Objectives

This study embarks on four distinct objectives as follows:

1. To investigate the effect of process condition (temperature and time) of double boiling treatment on physicochemical properties and brown pigment of *Kelulut* honey.
2. To determine the optimum experimental conditions of double boiling treatment for *Kelulut* honey processing by using Response Surface Methodology (RSM).
3. To determine the effect of the optimum double boiling condition (60 °C, 35 minutes) on the physicochemical and antioxidant properties of three different sources of *Kelulut* honey.
4. To evaluate a preliminary study on the storage effect on physicochemical and antioxidant properties of three different sources of *Kelulut* honey at room temperature (±27 °C) and cold temperature (±4 °C) after four weeks duration.

1.4 Research Scopes

The scope of the study is;

1. For a preliminary study, *Kelulut* honey has been treated using double boiling treatment (at 30, 60 and 90 °C for 10, 30 and 60 minutes) and conventional thermal processing (at 30, 60 and 90 °C for 10, 30 and 60 minutes). The pH, moisture content, total solids, total soluble solids and brown pigment after respective treatment were evaluated.
2. The optimum condition for double boiling treatment is assessed using Response Surface Methodology (RSM). Temperature at 30, 45 and 60 °C for 10, 35 and 60 minutes of treatment time were assessed and the optimum condition was further used in the study.
3. Changes in quality after treatment is evaluated based on physicochemical properties (pH, moisture content, total soluble solid (TSS), total solid, reducing sugar, diastase activity, free acidity, colour and hydroxymethylfurfural content (HMF)) and antioxidant properties (DPPH analysis, total phenolic content and brown pigment formation).

4. *Kelulut* honey processed at optimized condition (60 °C for 35 minutes) are tested for its storage effect on physicochemical and nutritional properties during four weeks at room temperature (± 27 °C) and cold temperature (± 4 °C) respectively.

CHAPTER 2

LITERATURE REVIEW

2.1 *Kelulut* Honey

Recognized as the bees from the tribe of Meliponini, stingless bee honey is different from the normal honey bee belonging to the tribe *Apini* with a single genus, *Apis*. Under the tribe *Meliponini,* they are further diverged into five other genera namely *Melipona, Trigona, Meliponula, Dectylurina* and *Lestrimelitta* (Baharuddin *et al*., 2014). In Malaysia, the stingless bees that can be found are from the genus *Trigona* spp, locally known as *Kelulut* (Baharuddin *et al*., 2014). The number of known stingless bee species worldwide is estimated to be around 400 to 500 while more than 30 species were found in Malaysia. Malaysian Agricultural Research and Development Institute (MARDI) stated that there are at least 11 out of 15 species of stingless bees have high potential to be domesticated in Malaysia including *Heterotrigona itama*, *Geniotrigona thoracica*, *Tetragonilla atripes* and *Tetrigona peninsularis*. In general, *Kelulut* honey produced by stingless bee is less viscous, darker in color and has strong acidic flavor as compared to other honey (Lye, 2015). Recently, *Kelulut* honey has been attracting beekeepers attentions as well as researchers and public due to their benefits in health (Mail, 2016).

Generally, honey bees produce honey from nectar of flowers, and secretion of living parts of plants (Codex Alimentarius Commission, 2001). *Kelulut* bees are much smaller in size which is about 3 to 5mm, and had a slimmer body that is totally different from the usual yellow and black honeybees (Tan, 2012). This criterion gives benefits in collecting nectar from flowers to the deepest space hence more vitamins and minerals were found. This is in accordance with previous study reported by Norjihada

Izzah *et al*. (2016) that showed a higher phenolic acid and flavonoid content was recorded in *Kelulut* honey as compared to Tualang honey (*Apis dorsata*). Moreover, stingless bee honey is twice as nutritious as ordinary honey (Mail, 2014).

2.1.1 Floral sources of *Kelulut* honey

Since the last decades, honey bees (*Apidae*, *Apini*), bumble bees (*Apidae*, *Bombini*), and stingless bees (*Apidae*, *Meliponini*) are proved to apply a system to keep foragers from visiting recently depleted flowers by using repellent scent marks (Núñez, 1967; Giurfa and Núñez, 1992; Giurfa, 1993; Goulson *et al*., 1998; Jane *et al*., 1998; Jane *et al*., 2001; Williams, 1998; Goulson *et al*., 2001). The same application is important in marking rich food sources (Ferguson and Free, 1979; Cameron, 1981; Schmitt and Bertsch, 1990; Villa and Weiss, 1990; Hrncir *et al*., 2004). This thus ensure a more efficient good gathering process by using lesser time on the same non-worthy flowers and more time on searching for high quality flowers (Schmitt and Bertsch, 1990; Martin Giurfa and Núñez, 1992). These researches on stingless bees and their foods have given the hints on importance of floral sources studies.

Silva *et al* (2013) has stated that native bee species (known as indigenous bees), stingless bees or *Meliponini* bees are common visitors in a biome composed by botanical species that adapted to tropical climates. Meanwhile, a study on Brazilian stingless bee honey has proven that the physicochemical properties the honey was affected by its floral sources (Sousa *et al*., 2016). The affected parameters were color, acidity, sugar profile, ash and proline content in the honeys where no differences were perceived for honeys from the same floral source although it is produced by different bee species. This findings may due to the stingless bees' mechanism on flower constancy that is determines by flower scents; proven by Judith *et al*. (1998). The same study also indicated that stingless bees displayed a distinct preference for a single floral scent (such as flower volatiles or compounds emanating from carcasses), where 78-87% of working bees returning to flowers of same floral scent during foraging. Therefore, this explains the uniqueness of stingless bee honey (which is *Kelulut* honey in this study) on its distinct taste and health benefits depending on the floral sources.

In this study, the types of *Kelulut* honey have been classified into three which are Coconut, Multifloral and Wild Flowers. The first type was collected from a bee farm in Kampung Bintang, Batu Pahat, Johor where the main floral source in this one-

acre farm is Coconut tree (*Cocos nucifera*), thus the reason it is classified as Coconut honey (*Kelulut-C*) (Figure 2.1) It has a sweet smell and a sweet taste with a light brown color. It is the most fluidity if observed through naked eyes. Meanwhile, the other two were collected in an approximately two-acres *Kelulut* bee farm in Serdang, Selangor. Although they are in the same farm, each of them is located two kilometers from the other. The Multifloral (*Kelulut-M*) honey was collected in the farm where guava and cinnamon were mainly planted in the area of 4000 square meters (Figure 2.2). Following the floral source, the taste gives a hint of spice and sour. It is visible that *Kelulut-M* has a darker color with thicker fluidity. Thirdly, Wildflowers honey (*Kelulut-W*) is collected at the sides of the farm where there are mainly wildflowers around the hives (Figure 2.3). It gives a more unique smell and taste as compared to *Kelulut-C* and *Kelulut-M* that gives fruity taste. The honey types are named as such only for the purpose of this study and these are not classified as standardized names for other *Kelulut* honey.

Figure 2.1: A *Kelulut* bee farm in Kampung Bintang, Batu Pahat, Johor where the main floral source in this one-acre farm is Coconut tree (*Cocos nucifera*).

Figure 2.2: A *Kelulut* bee farm in Serdang, Selangor where the main floral source in this 4000 square meters farm is guava and cinnamon.

Figure 2.3: A *Kelulut* bee farm in Serdang, Selangor where the main floral source in this 4000 square meters farm is wildflowers.

2.1.2 Stingless bee species and nest constructions

Bees play an important role in the ecosystem as they are said to be the main agent for pollinating many species of native and cultivated plants. Bees are also functioning to ensure the maintenance of genetic variability, productivity and quality of many fruits (Avila *et al.*, 2018). Stingless bees are a group of small to medium-sized bees, approximately 4mm of body length and are smaller compared to the normal honey bees (Hrncir, Jarau, & Barth, 2016). Stingless bees are commonly used as commercial pollinators. These bees are equipped with vestigial stings and are usually found in tropical and subtropical parts of the world. The absence of functional stings which

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